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Retinoic acid-induced downmodulation of telomerase activity in human cancer cells

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Abstract

Most human cancers express telomerase but its activity is highly variable and regulated by complex mechanisms. Recently, several studies have suggested that retinoic acid (RA) downregulates telomerase activity and that this effect could be a major determinant of its therapeutic activity. To elucidate possible mechanisms of RA-mediated downmodulation of telomerase activity, we measured the kinetics of concentration changes of several transcription regulators by using standard biochemical techniques at low (10 μM) and high (100 μM) RA concentrations. We further evaluated the global impact of the RA treatment on gene expression profiles using microarray. It was found that the kinetics of c-Myc correlates most closely with the telomerase activity suggesting in agreement with previous studies that this protein is a major intermediate of the RA-induced downregulation of telomerase activity. Other telomerase regulators as Sp1 and Mad1 did not exhibit significant correlation. The dominant role of c-Myc in RA-induced telomerase downmodulation is confirmed by microarray data. Additionally, a number of proteins were found as possible correlates of telomerase activity by microarray analysis. These data suggest a complex interplay between c-Myc and other proteins that may be important determinants of the RA effects on telomerase activity in human cancer cells. The complex mechanism through which telomerase activity is controlled during differentiation and cancer transformation is also reflected.
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Keywords: Telomerase regulation; Retinoic acid; c-Myc

Introduction

Telomerase is an RNA-dependent ribonucleoprotein polymerase that elongates telomeric repeats at the chromosome ends and may have other functions that are currently under investigation (Greider and Blackburn, 1989; Fu et al., 2002). Most human cancers express telomerase but its activity is highly variable and regulated by complex mechanisms (Shay and Bacchetti, 1997; Kyo and Inoue, 2002; Cong et al., 2002; Ducrest et al., 2002). The transcriptional regulation of the expression of the human telomerase catalytic subunit with reverse transcriptase activity (hTERT) appears to be a major determinant of the telomerase activity regulation (Ducrest et al., 2001; Kyo and Inoue, 2002). The hTERT promoter does not contain TATA box, and the 200- to 400-bp region proximal to the transcription initiation site is responsible for most of its transcriptional activity (Cong et al., 1999; Horikawa et al., 1999; Takakura et al., 1999). Multiple E-boxes, Sp1, Ets, and other binding sites for transcription factors are located in this core promoter region. c-Myc binds to these E-boxes through heterodimer formation with Max proteins and directly activates the hTERT transcription (Wu et al., 1999; Greenberg et al., 1999). Binding of the c-Myc antagonists, Mad proteins, as Mad/Max complexes decreases the

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activity of the hTERT promoter (Gunes et al., 2000; Kyo et al., 2000; Xu et al., 2001). Sp1 also binds to the core promoter and activates hTERT transcription (Kyo et al., 2000). Although overexpression of c-Myc is frequently observed in a wide variety of tumor types, and in some cases expression levels of c-Myc and Sp1 correlate with the levels of telomerase activity at different stages of transformation (Kyo et al., 2000), some tumors lack c-Myc overexpression despite the presence of telomerase activity. Sp1 protein is abundant in some types of normal cells which do not have high telomerase activity. Thus, the wide divergence of telomerase activity in cancer cells and the cancer-specific telomerase activation may require additional factors yet to be discovered.

The human leukemic U937 cell line has been a key tool for studying cancer, HIV infections, and cellular differentiation. It has been previously found through limiting dilution cloning that plus U937 subclones support HIV replication efficiently and minus clones do not. Lysosomal serine proteases, such as elastase, cathepsin G, and proteinase 3 which can process the p65 subunit of NF-κB, were present exclusively in the minus clones, in certain subclones of THP-1 and HL-60 cells, and in primary monocytes, but not in the plus clones. Compared to plus clones, HIV entry and the formation of the trimolecular complex between HIV gp120, CD4, and CXCR4 is impaired in minus clones. Such impairment of the gp120–CD4–CXCR4 complex formation has also been observed in macrophages. These observations, as well as the larger size, higher adherence, and slower growth rate of the minus clones, indicate that minus clones exhibit more mature phenotype than plus clones.

Telomerase activity declines during cell maturation in humans. Chemotherapy agents that result in differentiation of leukemic cells, such as retinoic acid, also result in inhibition of telomerase activity. Plus clones have more telomerase activity than minus cells in a ratio of 100 to 1, and on the basis that telomerase activity decreases as cells mature, minus clones are more differentiated and prone to apoptosis than plus clones (Xiao et al., 2002). These properties make plus and minus clones excellent candidates for a modeling system to study the regulation of telomerase and other differentiation-related genes in leukemic cells.

Materials and methods

Cells lines

The human promonocytic lymphoma U937 line (Sundstrom and Nilsson, 1976) clones 10 (plus clone) and 17 (minus clone) were provided by H. Moriuchi (NIAID, NIH, Bethesda, MD) and propagated in RPMI 1640 with 10% FCS (Moriuchi et al., 1997). The human prostate cancer cell lines PC3 and DU145, as well as the human breast cancer cell lines BT549 and MDA-MB435, were obtained through the Biological Test Branch, DTP, NCI. The same RPMI with 10% FCS medium was used to propagate these cancer cell lines.

Antibodies and assay kits

Antibodies to c-Myc (N-262), Mad1 (Fl-221), and Sp1 (1C6) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For quantitative telomerase activity assay, the $TRAP_{EZE}$XL kit was purchased from Intergen (Purchase, NY). Genomic DNA was isolated using the Puregene DNA isolation kit from Gentra (Research Triangle, NC).

Telomerase activity assay

Cells were washed once with ice-cold PBS and their numbers counted. Cell pellet was either lysed immediately or frozen at −80°C until use. Telomerase activity was determined quantitatively by using the fluorescence-based $TRAP_{EZE}$ XL Telomerase Detection Kit from Intergen. All experimental steps, calibration, and quantitations were carried out according to the manufacturer's protocol. The fluorescence signal was captured using the CytoFlour series 4000 fluorocytometer from PerSeptive Biosystems.

Levels of mRNA expression measured by Affymetrix genechips

Expression levels of genes in plus and minus leukemic clones under the treatment of retinoic acid were measured via microarrays. Labeled, single-stranded oligonucleotides from U937 leukemic plus and minus clones under treatment with retinoic acid were extracted at times 0, 6, 24, and 48 h and then allowed to hybridize in a solution with U95 genechips at a precise temperature to prevent nonspecific binding. After the hybridization period, the microarrays were washed and scanned to obtain expression levels of genes. In both plus and minus clones, the same 12,625 genes were measured for expression levels at time points 0, 6, 24, and 48 h for comparison. Extensive data mining of the signals of gene expression levels in each type of clone (plus or minus) was then required to filter and analyze results.

Normalization of the microarray data

Because the data are obtained from different experiments, normalization is required to enable direct comparison between expression data gathered from different time points, genes, and clones; changes in RNA concentrations of the leukemic clones used to hybridize the probes on the genechip during the actual experiments could cause relative differences in expression levels. The raw expression data directly collected from the genechips after scanning were normalized using the global normalization technique

(Microarray Suite 5.0, Affymetrix), with respect to the model/baseline array of parental U937 cells.

Preliminary data filtering

Because of the massive data set, not all 12,625 genes from each set were studied, and preliminary filtering was developed to hone in on statistically significant genes. An algorithm of statistical significance in the Microarray Suite 5.0 (Affymetrix) filtered the gene expressions on a basic level, assigning significance levels in degrees of presence (P—present, M—marginal, A—absent) to every gene at all four expression time points. Genes in both plus and minus clones with more than one time point marked to be statistically absent or marginal were removed from the study.

Background error

A threshold was calculated to counter any errors due to experimental background noise. To avoid the overinterpretation of low signals yielding high relative changes through fluctuations by chance, the following threshold was used (to designate the level at which gene expression would be designated as noise): $\mu + 2\sigma$, where μ and σ are the mean and standard deviation of expression levels of plus and minus genes which have absent calls at all four time points. Using this formula, the noise threshold was calculated to be 128.1, which means that genes with expression levels less then 128.1 at all four time points were removed from the study. Through filtering, 3254 genes remained in the plus set and 5099 different genes remained in the minus set.

Clustering

A *k*-means clustering algorithm was used to investigate trends in the dynamics of genes, as well as to relate the patterns to biological meaning. 8354 gene dynamics for plus and minus clones were clustered in combination using J-Express software (Molmine). The *k*-means clustering was initiated on a random seed with a maximum 200 iterations. Before clustering, gene expression levels were adjusted by dividing each absolute expression level by average value of four expressions (at 0, 6, 24, and 48 h). Four optimal clusters ($N = 4$) best conveyed the distinct dynamic groups of the gene profiles in this experiment.

Calculations of changes in the expression levels

To quantify the relative change for comparison, fold changes were calculated from the endpoints time 0 and time 48 as a fold change with minus sign if gene's expression level at time 48 h was lower than its expression level at time 0 h. A gene was marked positively regulated if its fold calculation was positive.

Results

Downregulation of telomerase activity: effects of cell type and retinoic acid concentration

It has been previously shown that retinoic acid can downregulate telomerase activity for several cell lines. Here we extend these observations by using prostate and breast cancer cell lines. As is shown in Figs. 1 and 2, the retinoic acid significantly downregulates telomerase activity in several prostate and breast cancer cell lines including PC3, DU145, BT549, and MDA-MB435. At relatively low

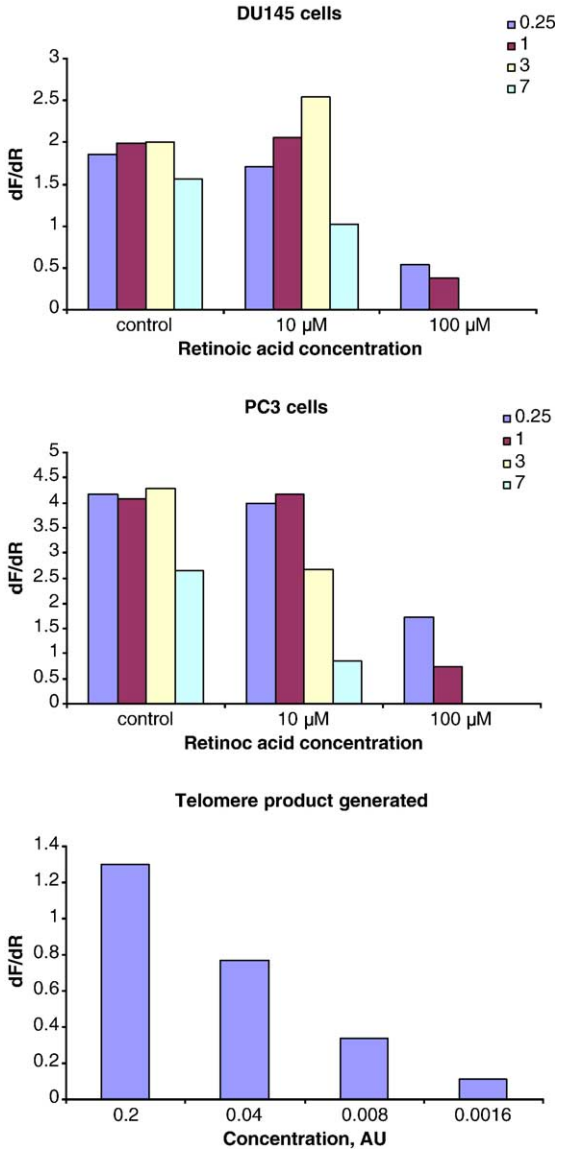

Fig. 1. Two prostate cancer cell lines, PC3 and DU145 with different endogenous telomerase activity, were subjected to retinoic acid treatment at different concentrations (10 and 100 μM) for different times (6 h, 1, 3, and 7 days). Cells were collected and telomerase activity was analyzed. Note that at days 3 and 7, 100 μM retinoic acid-treated cells died completely and no telomerase activity assay could be done. Synthesized telomeric repeat (TPG) was included in the TRAP assay as a positive control and standard.

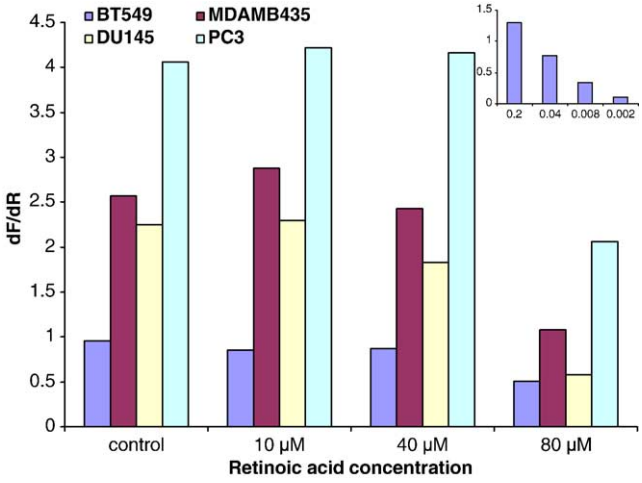

Fig. 2. Two breast cancer cell lines with different endogenous telomerase activity BT549 and MDA-MB435 and two prostate cell lines DU145 and PC3 were treated with different concentrations of retinoic acid (10, 40, and 80 μM) for 6 h and the telomerase activity was analyzed. Again, TPG was included as a positive control and standard.

concentrations of retinoic acid, the time required to reach significant levels of telomerase activity downregulation was of the order of days. Interestingly, an increase in the retinoic acid concentration to 80 μM resulted in a dramatic down-regulation of telomerase activity in a very short period of time, 6 h (Fig. 2). These results suggest that the mechanism leading to telomerase activity downregulation is likely to be universal for many cancer cells and that an increase in the retinoic acid concentration probably reduces telomerase activity by mechanisms not related to transcriptional regulation because of the long half-life of the telomerase. Low concentration of RA was therefore used to treat cells in microarray studies to exclude gene regulation mechanisms not directly related to transcriptional control.

c-Myc is the key regulator in retinoic acid-induced downregulation of telomerase

To explore possible mechanisms of telomerase regulation by retinoic acid, we measured the kinetics of telomerase activity, and cell proliferation and apoptosis, in parallel with levels of expression of several key proteins that were implicated in the transcriptional regulation of the telomerase activity: c-Myc and its partner Mad1, and Sp1. For comparison, we also measured the same parameters for two other reagents that affect telomerase activity: sodium butyrate and TPA. We used two subclones of the U937 cell line which exhibit high (plus clone 10) and low (minus clone 17) telomerase activity. The results suggest that c-Myc kinetics of concentration changes most closely correlates with telomerase activity (Fig. 3). Apoptosis increased with a decrease in the telomerase activity. These results suggest that for these leukemic cell clones, c-Myc is a key regulator of telomerase activity and a major determinant of the effect of retinoic acid leading to downregulation of telomerase activity. Butyrate and TPA appeared to regulate the telomerase activity through the same c-Myc pathway, as the correlation between c-Myc level and telomerase activity was clear during treatments.

Microarray analysis of retinoic acid-induced downregulation of telomerase activity

In an initial attempt to identify proteins that could be potentially mediating some of the retinoic acid effects, we measured mRNA profiles at different time points by Affymetrix microarrays in plus and minus U937 clones and analyzed the results. Four types of distinct dynamics emerged from the genes of the leukemic plus and minus clones through clustering (Fig. 4). 2593 genes from plus and minus clones displayed constant expression (CE); 2335 genes displayed downregulation (DR); 794 genes displayed a delayed upregulation which initiated after 24 h of constant expression (UR); and 2632 genes displayed an initial upregulation for 24 h followed by downregulation for the next 24 h (UDR). Upon analysis of locations of key telomerase regulatory genes c-Myc, Ets, ID1/2/3, and Max in these leukemic clones under the anti-cancer treatment retinoic acid, it was found that many regulators of c-Myc were downregulated. This fact correlates well with fold calculations and cluster assignments; many of the regulators of c-Myc yield negative folds of two or more (Table 1), signifying the overall downregulation from time 0 to time 48 h. Max interacting proteins display a slight trend in upregulation under the anti-cancer treatment in accordance to fold calculations (Table 2). The increased presence of proteins which antagonize c-Myc for Max represses the proliferation that the Max/Myc complex promotes. Max proteins themselves in both clones displayed well over 3-fold upregulation and were clustered in the delayed upregulation cluster. ID1/2/3 proteins and their associating factors were marked as genes which experienced delayed but eventual upregulation. Fold calculations (Table 3) show that the anti-cancer treatment (retinoic acid) effectively increased the presence of cancer repressing proteins as ID1/2/3 proteins, which negatively regulate differentiation by controlling growth promoters as the ETS family (Table 4). The support for the control of cell proliferation by genes such as ID1/2/3 and Max suggests a mechanism of cell proliferation inhibition when under anti-cancer treatment. Overall, only a select number of gene products were modulated more than thirty-fold, about 200 gene products were modulated more than fivefold, and about 2000 gene products modulated more than twofold. Most genes whose expression was downregulated more than tenfold comprised of genes responsible for inflammatory processes, which may suggest that the retinoic acid is effecting a change in the immune system (Table 5). Other genes that displayed significant downregulation (more than 15-fold) included a CTP synthase, known for its key role in tumorigenesis, and genes associated with c-Myc (more than 5-fold). Genes of Cluster C (UR) activate expression after constancy at the same time (24 h) that genes in Cluster D

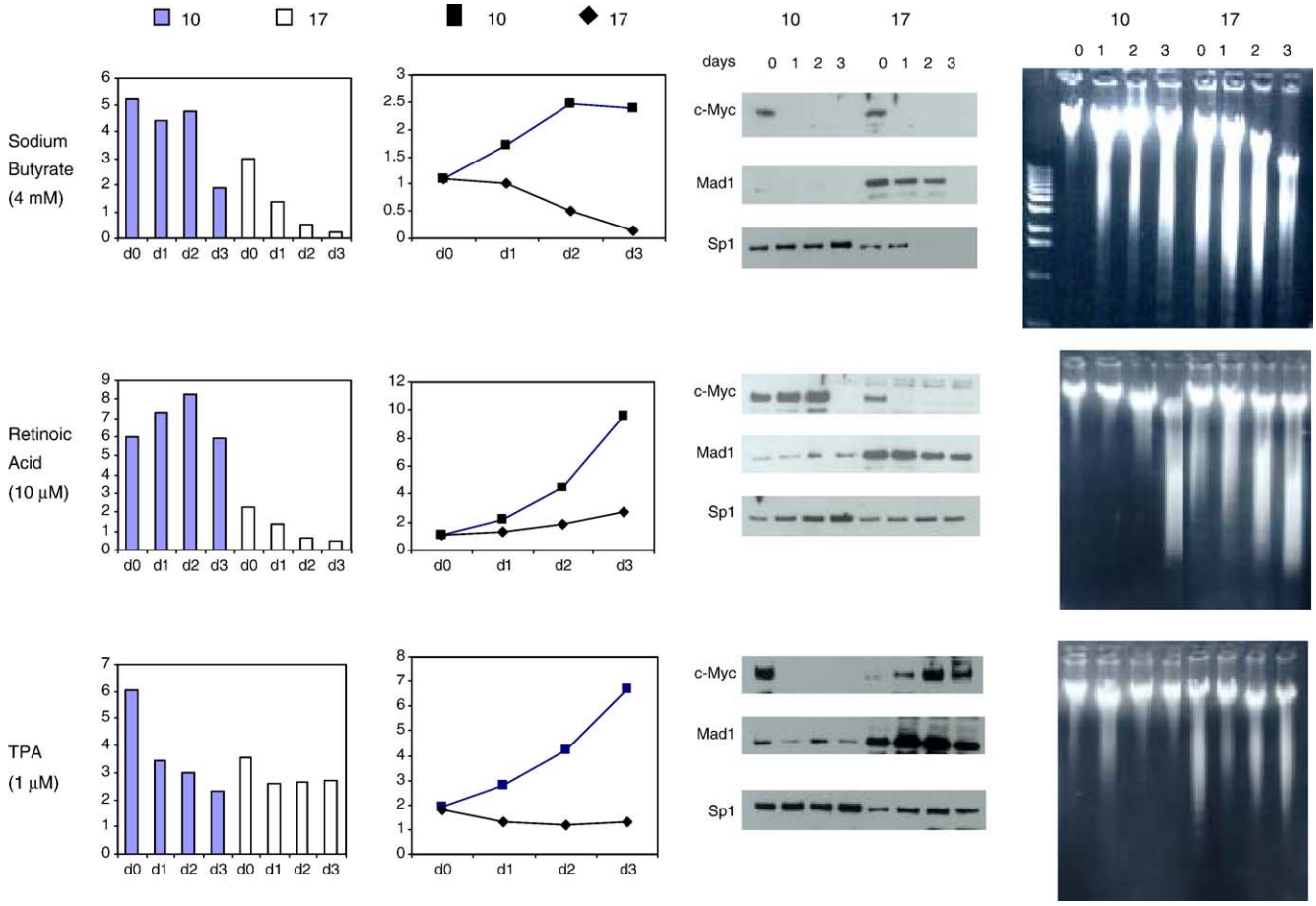

Fig. 3. The effect of differentiation agent treatment on U937 plus and minus clones. Shown in the figures are the telomerase activity (left column), growth curves (second from left column), expression level of key transcriptional factors (third from left column), and the apoptosis induced (the last column) of the plus (Horikawa et al., 1999) and minus (Kyo et al., 2000) clones after treatment with various differentiation agent at indicated concentration for the described period of time.

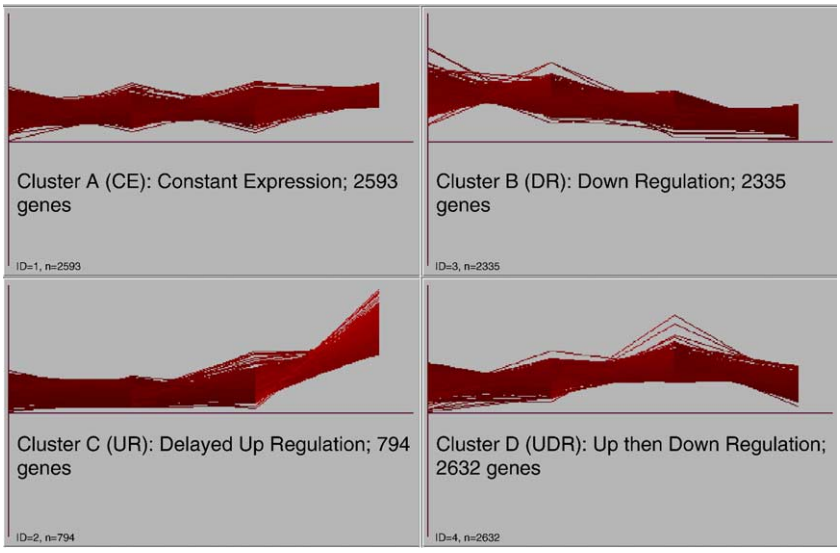

Fig. 4. Clustering of 8354 plus and minus gene expression filtered values in combination using the Euclidean measure of distance in *k*-means algorithm. Clustering was initiated on a random seed with a maximum limit for 200 iterations. *x* axis displays the time, defined at time 0, 6, 24, and 48 h; *y* axis displays relative gene expression level (raw expression level divided by average expression level of four time points of a given gene).

Table 1
Genes associated with c-Myc

Affymetrix notation [Genbank notation]	Clone	Dynamics cluster	Fold change
Transcriptional regulation of c-myc expression			
1521_at [X17620]	Minus	Downregulation	−10[a]
1521_at [X17620]	Plus	Up- then downregulation	−2
1985_s_at [X73066]	Minus	Downregulation	−7
1985_s_at [X73066]	Plus	Constant expression	2
39073_at [AL038662]	Minus	Downregulation	−9
39073_at [AL038662]	Plus	Up- then downregulation	−1
39860_at [U05040]	Minus	Downregulation	−7
39860_at [U05040]	Plus	Constant expression	−1
Transcriptional repressor of c-myc expression			
1817_at [D89667]	Minus	Up- then downregulation	−1
1817_at [D89667]	Plus	Up- then downregulation	−1
2035_s_at [M55914]	Minus	Downregulation	−2
2035_s_at [M55914]	Plus	Constant expression	1
32075_at [D89859]	Plus	Constant expression	1
35319_at [U25435]	Minus	Downregulation	−2
35319_at [U25435]	Plus	Up- then downregulation	−1
Binds to c-Myc replication origin/transcriptional enhancer site			
31671_at [D82351]	Plus	Up- then downregulation	−1
31672_g_at [D82351]	Minus	Up- then downregulation	−1
31672_g_at [D82351]	Plus	Up- then downregulation	−2
33867_s_at [X77494]	Minus	Up- then downregulation	1
33867_s_at [X77494]	Plus	Up- then downregulation	1

[a] Folds were calculated from the endpoints time 0 and time 48 by dividing the greater endpoint by the lesser endpoint and multiplied by −1 if a gene's expression level at time 48 was lower than its expression level at time 0.

(UDR) experience a turning point in expression; this counter exchange insinuates a strict, delicate balance in gene expression and regulation. Most of up- and downregulated genes (represented in Tables 5 and 6, more than 5-fold of changes) belong to the minus clone: 71% for upregulated genes and 91% for downregulated genes. Significant upregulation (more than 15-fold) was shown in 13 genes (3 for Plus and 10 Minus clones, respectively). Among them: transglutaminase 2, overexpression in minus but not in plus clone (extracellular enzyme playing an important role in many intracellular signaling cascade and extracellular assembly processes); CD38 antigen (p45) regulating cell proliferation and activation was upregulated more than 80 times both in minus and plus clones; Rac2, regulator of mitogen-induced cytoskeletal changes essential for membrane ruffling, 24-fold in minus clone; and hexokinase 3, involved in glucose methabolism, 36-fold upregulation in minus clone.

Table 2
Genes associated with MAX

Affymetrix notation [Genbank notation]	Clone	Dynamics cluster	Fold change
MAX protein; forms heterodimers with MYC, MAD and regulates gene expression			
1981_s_at [X60287]	Minus	Delayed upregulation	6
1981_s_at [X60287]	Plus	Delayed upregulation	4
MAX dimerization protein 5			
34706_at [AB011090]	Plus	Downregulation	−1
MAX interacting protein; heterodimerizes with MAX to antagonize MYC activity and negatively regulate MYC function			
35145_at [X96401]	Minus	Up- then downregulation	−1
35145_at [X96401]	Plus	Downregulation	−2
39072_at [L07648]	Minus	Constant expression	2
39072_at [L07648]	Plus	Constant expression	2
654_at [L07648]	Minus	Constant expression	1
654_at [L07648]	Plus	Constant expression	2

Table 3
Genes associated with Id proteins

Affymetrix notation [Genbank notation]	Clone	Dynamics cluster	Fold change
Id1 protein; negatively regulates cell differentiation			
36618_g_at [X77956]	Minus	Delayed upregulation	10
36618_g_at [X77956]	Plus	Delayed upregulation	4
36617_at [X77956]	Minus	Constant expression	5
36617_at [X77956]	Plus	Up- then downregulation	1
Id2 protein; negatively regulates cell differentiation			
41215_s_at [D13891]	Minus	Downregulation	−2
41215_s_at [D13891]	Plus	Constant expression	−1
Id3 protein; inhibits DNA-binding of E2A-containing complexes			
37043_at [AL021154]	Plus	Delayed upregulation	3

Discussion

The regulation of telomerase activity is a multifactorial process that involves a number of transcriptional factors (Kyo and Inoue, 2002). Though c-Myc has been established as the dominant regulator, other factors reported to regulate telomerase activity have far more ambiguous roles and mechanisms. Further studies are required to clarify the molecular details of telomerase regulation mediated by the other factors although it is almost certain that these effects are dependent on the interplay with other transcription factors.

Table 4
Genes associated with ETS

Affymetrix notation [Genbank notation]	Clone	Dynamics cluster	Fold change
ETS domain transcription factor			
31845_at [U32645]	Minus	Constant expression	2
33275_at [AB016194]	Minus	Constant expression	−1
33275_at [AB016194]	Plus	Constant expression	1
38491_at [U11732]	Plus	Constant expression	2
40067_at [M82882]	Minus	Downregulation	−2
40067_at [M82882]	Plus	Up- then downregulation	−2
41425_at [M98833]	Minus	Constant expression	2
41425_at [M98833]	Plus	Constant expression	1
507_s_at [U43189]	Plus	Up- then downregulation	1
Transcriptional repressor; suppresses ets-induced transformation			
1242_at [U15655]	Minus	Constant expression	−1
1242_at [U15655]	Plus	Delayed upregulation	2
38996_at [U15655]	Minus	Downregulation	−1
38996_at [U15655]	Plus	Delayed upregulation	2

Table 5
Downregulated genes

Affymetrix notation [Genbank notation]	Clone	Description	Fold change
875_g_at [M26683]	Minus	Cytokine genes	−44
34375_at [M28225]	Minus	Cytokine genes	−22
32441_at [X52142]	Minus	Enzyme cytidine-5-prime-triphosphate synthetase; plays a key role in cell growth, development, and tumorigenesis	−17
37458_at [AJ223728]	Minus	Cdc45 protein; key role in initiation of DNA replication	−12
40490_at [U41387]	Minus	[RefSeq summary:] DEAD box proteins	−12
34882_at [Y12065]	Minus	Protein involved in pre-rRNA processing	−11
37324_at [X01060]	Minus	Transferrin receptor	−11
41085_at [AF025840]	Minus	Subunit 2 of DNA polymerase epsilon	−11
33802_at [Z82244]	Minus	Heme oxygenase	−10
1521_at [X17620]	Minus	Transcription factor and nucleoside diphosphate kinase; has a role in the transcriptional regulation of c-*myc* expression	−10
37724_at [V00568]	Minus	[Proteome summary:] transcription factor; activates and represses expression of target genes	−10
40982_at [AA926957]	Minus	*Homo sapiens* cDNA	−10
1979_s_at [X55504]	Minus	Proliferation-associated antigen	−9
39073_at [AL038662]	Minus	Transcription factor and nucleoside diphosphate kinase; has a role in the transcriptional regulation of c-*myc* expression	−9
36460_at [AF008442]	Minus	Subunit of RNA polymerase I	−9
1973_s_at [V00568]	Minus	Transcription factor; activates and represses expression of target genes	−8
549_at [S80343]	Minus	Aminoacyl-tRNA synthetases	−8
1179_at [None]	Minus	Heat shock protein	−8
1516_g_at [None]	Minus	Rad2	−8
41600_at [U59435]	Minus	Strongly similar to murine Plfap	−8
36684_at [M21154]	Minus	*S*-adenosylmethionine decarboxylase	−8
33165_at [AL041879]	Minus	*Homo sapiens* cDNA	−8
1985_s_at [X73066]	Minus	Transcription factor and nucleoside diphosphate kinase; has a role in the transcriptional regulation of c-*myc* expression	−7
34857_at [Z24724]	Minus	*Homo sapiens* poly-A site	−7
33252_at [D38073]	Minus	Initiation of DNA replication	−7
37646_at [D26018]	Minus	Subunit of DNA polymerase delta 3	−7
39677_at [D80008]	Minus	Human mRNA	−7
39860_at [U05040]	Minus	Stimulates expression of c-*myc* in undifferentiated cells	−7
41583_at [AC004770]	Minus	Flap endonuclease; double-stranded DNA 5′-3′ exonuclease	−7
36597_at [D21262]	Minus	Nucleolar phosphoprotein	−7
32186_at [M80244]	Minus	Sodium-independent neutral amino acid transporter	−7
39056_at [X53793]	Minus	Bifunctional polypeptide	−6
38384_at [X54199]	Minus	Trifunctional polypeptide	−6
41259_at [AI553745]	Minus	*Homo sapiens* cDNA	−6
40457_at [AF038250]	Minus	Strongly similar to murine Sfrs3	−6
36135_at [U86602]	Minus	Has multiple predicted transmembrane domains	−6
1191_s_at [AB003102]	Minus	Non-ATPase subunit	−6
39748_at [AL050021]	Minus	*Homo sapiens* mRNA	−6
34765_at [D13645]	Minus	*Homo sapiens* mRNA	−6
39269_at [L07541]	Minus	Activator of DNA polymerases	−6
41855_at [AF030424]	Minus	Catalytic subunit of histone acetyltransferase	−6
39791_at [M23114]	Minus	Slow twitch cardiac muscle Ca2+-ATPase	−6
41060_at [M74093]	Minus	Cyclin family protein	−6
39379_at [AL049397]	Minus	*Homo sapiens* mRNA	−6
32194_at [M37197]	Minus	Gene-specific transcriptional activation	−6
34818_at [X96381]	Minus	DNA binding protein of the Ets oncoprotein family	−6
1154_at [J02645]	Minus	Alpha subunit of translation initiation factor 2	−6
262_at [M21154]	Minus	*S*-adenosylmethionine decarboxylase	−6
33373_at [AL049951]	Plus	*Homo sapiens* mRNA	−6
229_at [M37197]	Minus	Gene-specific transcriptional activation	−6
35249_at [AF091433]	Minus	Cyclin family protein	−6
37379_at [X81789]	Minus	Spliceosome-associated protein 3a	−6
430_at [X00737]	Minus	Nucleoside phosphorylase	−6
41213_at [X67951]	Minus	Strongly similar to murine Paga	−6

Previous studies have provided evidence that c-Myc and Sp1 are involved in hTERT transcriptional regulation (Kyo and Inoue, 2002). However, in some cancer cells, apparently telomerase activity can be regulated independently on c-Myc (Drissi et al., 2001). Many cancer cell lines with undetectable c-Myc exhibited high telomerase activity and vice versa. This suggests that although c-Myc plays an

Table 6
Upregulated genes

Affymetrix notation [Genbank notation]	Clone	Description	Fold change
38404_at [U25165]	Minus	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	375
40323_at [AF070585]	Plus	CD38 antigen (p45)	95
40323_at [AF070585]	Minus	CD38 antigen (p45)	88
36372_at [AL034396]	Minus	Hexokinase 3 (white cell)	36
35051_at [AF007136]	Minus	Carbonic anhydrase VI	30
31431_at [U12255]	Minus	Fc fragment of IgG, receptor, transporter, alpha	25
32737_at [AF050640]	Minus	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	24
36151_at [X79353]	Minus	Similar to vaccinia virus *Hin*dIII K4L ORF	24
39825_at [W29115]	Minus	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1	22
34777_at [AA418080]	Plus	Adrenomedullin	19
37929_at [U11700]	Minus	Immunoglobulin superfamily, member 4	19
910_at [M19311]	Plus	Thymidine kinase 1, soluble	18
39827_at [AA477714]	Minus	HIF-1 responsive RTP801	17
38389_at [Z34975]	Minus	2′,5′-Oligoadenylate synthetase 1, 40/46 kDa	14
41017_at [AL050015]	Minus	Myosin binding protein H	14
38388_at [X04371]	Minus	2′,5′-Oligoadenylate synthetase 1, 40/46 kDa	14
38567_at [U82939]	Minus	CD1D antigen, d polypeptide	14
41191_at [AF091090]	Minus	Palladin	13
625_at [L78833]	Minus	Vesicle amine transport protein 1 homolog (*T. californica*)	13
33410_at [S66213]	Minus	Integrin, alpha 6	13
32737_at [AF050640]	Plus	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	13
35807_at [AL031681]	Minus	Cytochrome b-245, alpha polypeptide	13
33103_s_at [W27614]	Minus	Adducin 3 (gamma)	12
39827_at [AA477714]	Plus	HIF-1 responsive RTP801	12
35934_at [Y08683]	Plus	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52 kDa	12
33944_at [D31797]	Minus	Amyloid beta (A4) precursor-like protein 2	12
38430_at [U09759]	Minus	Fatty acid binding protein 4, adipocyte	12
1045_s_at [U34605]	Plus	V-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	12
1272_at [L22005]	Plus	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52 kDa	12
32612_at [AI278113]	Minus	Gelsolin (amyloidosis, Finnish type)	11
455_at [U66619]	Plus	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	11
41471_at [AL078641]	Minus	S100 calcium binding protein A9 (calgranulin B)	11
33097_at [U28694]	Minus	CCAAT/enhancer binding protein (C/EBP), epsilon	10
37074_at [AF028828]	Minus	Syntrophin, beta 1 (dystrophin-associated protein A1, 59 kDa, basic component 1)	10
36980_at [AF070649]	Minus	Proline rich 2	10
36618_g_at [S78825]	Minus	Inhibitor of DNA binding 1, dominant negative helix–loop–helix protein	10
625_at [L78833]	Plus	Vesicle amine transport protein 1 homolog (*T. californica*)	10
33989_f_at [U22961]	Plus	Testis enhanced gene transcript (BAX inhibitor 1)	10
31432_g_at [AL022727]	Minus	Fc fragment of IgG, receptor, transporter, alpha	9
40101_g_at [AB018315]	Minus	rho/rac guanine nucleotide exchange factor (GEF) 2	9
39825_at [W29115]	Plus	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1	9
41337_at [AI951946]	Plus	Amino-terminal enhancer of split	9
40081_at [D10040]	Minus	Phospholipid transfer protein	9
36979_at [U03105]	Plus	Solute carrier family 2 (facilitated glucose transporter), member 3	8
34959_at [M15059]	Minus	Fc fragment of IgE, low affinity II, receptor for (CD23A)	8
40390_at [AB007448]	Minus	Serine dehydratase	8
33411_g_at [AI535946]	Minus	Integrin, alpha 6	8
38363_at [AF068197]	Minus	TYRO protein tyrosine kinase binding protein	8
605_at [L78833]	Plus	Vesicle amine transport protein 1 homolog (*T. californica*)	8
41198_at [W27050]	Minus	Granulin	7
40439_at [AL080119]	Plus	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)	7
1974_s_at [X03563]	Plus	Tumor protein p53 (Li-Fraumeni syndrome)	7

(continued on next page)

Table 6 (*continued*)

Affymetrix notation [Genbank notation]	Clone	Description	Fold change
38259_at [AL050306]	Minus	Syntaxin binding protein 2	7
32893_s_at [AF053356]	Minus	Gamma-glutamyltransferase 2	7
35017_f_at [U61538]	Minus	Major histocompatibility complex, class I, J (pseudogene)	7
33106_at [AB020705]	Minus	Nuclear receptor subfamily 1, group H, member 3	7
34362_at [Z11793]	Minus	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	7
39077_at [U43286]	Plus	DR1-associated protein 1 (negative cofactor 2 alpha)	7
1224_at [X66363]	Plus	PCTAIRE protein kinase 1	7
33803_at [U43522]	Minus	Thrombomodulin	7
35829_at [AB002368]	Minus	Immunoglobulin superfamily, member 4	7
40100_at [U72206]	Minus	rho/rac guanine nucleotide exchange factor (GEF) 2	7
36262_at [Z12173]	Minus	Glucosamine (*N*-acetyl)-6-sulfatase (Sanfilippo disease IIID)	7
32905_s_at [U21936]	Minus	Tryptase, alpha	7
394_at [X94629]	Plus	Bleomycin hydrolase	7
2045_s_at [M21536]	Minus	Hemopoietic cell kinase	7
40470_at [Y09048]	Minus	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	7
1671_s_at [L41913]	Plus	Mitogen-activated protein kinase 14	7
36629_at [Z50781]	Minus	Delta sleep inducing peptide, immunoreactor	7
34777_at [AA418080]	Minus	Adrenomedullin	7
40782_at [L36151]	Minus	Short-chain dehydrogenase/reductase 1	7
33236_at [AB018344]	Minus	Retinoic acid receptor responder (tazarotene induced) 3	7
37421_f_at [S71018]	Minus	Major histocompatibility complex, class I, F	7
239_at [M64231]	Minus	Cathepsin D (lysosomal aspartyl protease)	6
36586_at [Z11692]	Minus	Aryl hydrocarbon receptor interacting protein	6
33212_at [AF006751]	Minus	Ribosome binding protein 1 homolog 180 kDa (dog)	6
36933_at [AL035447]	Minus	*N*-myc downstream regulated gene 1	6
41300_s_at [W28608]	Minus	Integral membrane protein 2B	6
1217_g_at [X12794]	Minus	Protein kinase C, beta 1	6
32523_at [W29012]	Plus	Clathrin, light polypeptide (Lcb)	6
1780_at [M25269]	Minus	Gardner–Rasheed feline sarcoma viral (v-fgr) oncogene homolog	6
715_s_at [D87002]	Minus	Gamma-glutamyltransferase 1	6
39076_s_at [U41843]	Plus	DR1-associated protein 1 (negative cofactor 2 alpha)	6

Table 6 (*continued*)

Affymetrix notation [Genbank notation]	Clone	Description	Fold change
40332_at [U43842]	Minus	Opioid growth factor receptor	6
39280_at [AB002378]	Minus	trinucleotide repeat containing 5	6
35339_at [AI819948]	Minus	mel transforming oncogene (derived from cell line NK14)- RAB8 homolog	6
35807_at [AL031681]	Plus	Cytochrome b-245, alpha polypeptide	6
1358_s_at [U32659]	Minus	Interferon, alpha-inducible protein (clone IFI-6-16)	6
34378_at [AF082657]	Plus	Adipose differentiation-related protein	6
33724_at [U38291]	Plus	Breast cancer 1, early onset	6
39182_at [X66363]	Plus	Epithelial membrane protein 3	6
391_at [X89416]	Minus	Protein phosphatase 5, catalytic subunit	6
40545_at [AF047185]	Minus	Proline synthetase co-transcribed homolog (bacterial)	6
37148_at [U95626]	Minus	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	6
38397_at [AB002356]	Minus	Polymerase (DNA-directed), delta 4	6
1550_at [U19796]	Plus	Melanoma-associated antigen recognised by cytotoxic T lymphocytes	6
36287_at [X81420]	Minus	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	6
318_at [D64142]	Minus	H1 histone family, member X	6
34890_at [AI540958]	Plus	ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A, isoform 1	6
38998_g_at [M86707]	Minus	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1	6
1981_s_at [X68452]	Minus	MAX protein	6
374_f_at [Z84718]	Minus	D-dopachrome tautomerase	6
40514_at [AF035280]	Plus	Serologically defined breast cancer antigen 84	6
41735_at [AI808958]	Minus	KIAA0870 protein	6
34223_at [AC004770]	Minus	Colony stimulating factor 3 receptor (granulocyte)	6
1649_at [U61836]	Minus	Chromosome 20 open reading frame 16	6

important role in the regulation of the telomerase activity in cancer cells, other factors could be equally important. In this study using biochemical approach, we further confirmed the very important role of c-Myc and its relevant regulatory

factors in regulating telomerase activity. The roles of other factors are less clear. Among them, Sp1 did not show any correlation between its expression and telomerase activity for the cell lines with various treatments. We failed to detect any correlation in the NCI 60 cancer cell line panel either (data not shown). Another factor, Mad1, existed in a much higher level in minus cells. This agrees with its proposed role in downmodulating telomerase activity. However, the lack of change in its protein level during RA or other treatments seems to suggest that its roles in telomerase regulation are restricted. This calls for the careful examination of the interplay between those potential regulators that might be revealed by microarray data from cell lines undergoing differentiation either naturally or induced by drugs.

In analyzing the microarray data, we paid special attention to some of the factors that we showed in our previous studies that are involved in telomerase activity regulation. These factors include c-Myc associated factors, Ets and Id family members. Similar to the biochemical data, except for c-Myc associated factors, there is no clear-cut trend for other regulatory factors. However, some interesting patterns did occur.

As it is shown in Fig. 4, most of the genes were down- or up-then-downregulated (about 4967 genes) and the average dynamics for these groups of genes (average values of gene expression for all genes in group) were delayed for about 6 h. Upregulation for 794 genes was delayed for about 1 day which suggests more complex regulation. Two Id family members (Id1 and Id3) with upregulation of 3- to 10-fold belong to this delayed upregulation clusters. Since minus clones are supposedly more differentiated and mature than plus clones, gene expression dynamics from a later time in plus clones should resemble the early dynamics of corresponding genes in the minus clones. Another technique to study genetic interactions between telomerase regulating genes as c-Myc, Mad, Max, ID 1/2/3, and ETS is through the use of genetic network modeling. These genetic regulatory networks offer insightful and new information about mechanisms and relations of significant genes. On the contrary, the approach used in this particular study can help us to further elucidate the mechanisms of differentiation and telomerase regulation.

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References

Cong, Y.S., Wen, J., Bacchetti, S., 1999. The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. Hum. Mol. Genet. 8 (1), 137–142.

Cong, Y.S., Wright, W.E., Shay, J.W., 2002. Human telomerase and its regulation. Microbiol. Mol. Biol. Rev. 66 (3), 407–425 (table).

Drissi, R., Zindy, F., Roussel, M.F., Cleveland, J.L., 2001. C-Myc-mediated regulation of telomerase activity is disabled in immortalized cells. J. Biol. Chem. 276 (32), 29994–30001.

Ducrest, A.L., Amacker, M., Mathieu, Y.D., Cuthbert, A.P., Trott, D.A., Newbold, R.F., Nabholz, M., Lingner, J., 2001. Regulation of human telomerase activity: repression by normal chromosome 3 abolishes nuclear telomerase reverse transcriptase transcripts but does not affect c-Myc activity.

Ducrest, A.L., Szutorisz, H., Lingner, J., Nabholz, M., 2002. Regulation of the human telomerase reverse transcriptase gene. Oncogene 21 (4), 541–552.

Fu, W., Lu, C., Mattson, M.P., 2002. Telomerase mediates the cell survival-promoting actions of brain-derived neurotrophic factor and secreted amyloid precursor protein in developing hippocampal neurons. J. Neurosci. 22 (24), 10710–10719.

Greenberg, R.A., O'Hagan, R.C., Deng, H., Xiao, Q., Hann, S.R., Adams, R.R., Lichtsteiner, S., Chin, L., Morin, G.B., DePinho, R.A., 1999. Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. Oncogene 18 (5), 1219–1226.

Greider, C.W., Blackburn, E.H., 1989. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 337 (6205), 331–337.

Gunes, C., Lichtsteiner, S., Vasserot, A.P., Englert, C., 2000. Expression of the hTERT gene is regulated at the level of transcriptional initiation and repressed by Mad1. Cancer Res. 60 (8), 2116–2121.

Horikawa, I., Cable, P.L., Afshari, C., Barrett, J.C., 1999. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res. 59 (4), 826–830.

Kyo, S., Inoue, M., 2002. Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy? Oncogene 21 (4), 688–697.

Kyo, S., Takakura, M., Taira, T., Kanaya, T., Itoh, H., Yutsudo, M., Ariga, H., Inoue, M., 2000. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). Nucleic Acids Res. 28 (3), 669–677.

Moriuchi, H., Moriuchi, M., Arthos, J., Hoxie, J., Fauci, A.S., 1997. Promonocytic U937 subclones expressing CD4 and CXCR4 are resistant to infection with and cell-to-cell fusion by T-cell-tropic human immunodeficiency virus type 1. J. Virol. 71 (12), 9664–9671.

Shay, J.W., Bacchetti, S., 1997. A survey of telomerase activity in human cancer. Eur. J. Cancer 33 (5), 787–791.

Sundstrom, C., Nilsson, K., 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer 17 (5), 565–577.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., Inoue, M., 1999. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res. 59 (3), 551–557.

Wu, K.J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J., Dalla-Favera, R., 1999. Direct activation of TERT transcription by c-MYC. Nat. Genet. 21 (2), 220–224.

Xiao, X., Phogat, S.K., Sidorov, I.A., Yang, J., Horikawa, I., Prieto, D., Adelesberger, J., Lempicki, R., Barrett, J.C., Dimitrov, D.S., 2002. Identification and characterization of rapidly dividing U937 clones with differential telomerase activity and gene expression profiles: role of c-Myc/Mad1 and Id/Ets proteins. Leukemia 16, 1877–1880.

Xu, D., Popov, N., Hou, M., Wang, Q., Bjorkholm, M., Gruber, A., Menkel, A.R., Henriksson, M., 2001. Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. Proc. Natl. Acad. Sci. U. S. A. 98 (7), 3826–3831.